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Proteins localized to the cell membrane or secreted show great promise as therapeutic targets and diagnostic markers because of their easy accessibility. However, determining protein localization by traditional methods is a difficult process. A "feature" of membrane-bound and secreted proteins can be exploited to determine their membrane-bound status on a large scale. Because the mRNA transcripts of membrane-bound and secreted proteins are translated in polysomes bound to the endoplasmic reticulum (ER), they can be separated from their heavier cytosolic counterparts by sucrose gradient centrifugation. At the end of year one of this project, we have reproducibly separated the RNA of MCF7 cells into two fractions using this method. Realtime RT-PCR analysis of two test genes shows enrichment of the RNA encoding cytoplasmic GAPDH in the expected fraction, and an enrichment of the RNA encoding membrane-localized JAM in the membrane fraction. Although we have experienced some difficulty in increasing RNA yield, we are confident we will soon have enough RNA to hybridize to Affymetrix chips, so that we may continue this identification on a large scale. Combined with breast cancer expression and amplicon data, this could allow for the identification of potential novel membrane-bound and secreted drug targets and markers.

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Novel Membrane-Associated Targets for Diagnosis and Treatment of Breast Cancer

Introduction

Genomic methods of analysis, such as DNA microarrays and comparative genomic hybridization (CGH), have produced a wealth of data describing overexpressed genes and amplified chromosomal regions (amplicons) in breast cancer tumors and cell lines. Those genes encoding proteins localized to the cell membrane or secreted show great promise as therapeutic targets and diagnostic markers because of their easy accessibility to antibodies and potential presence in bodily fluids, respectively. Of the nearly 30,000 estimated genes in the human genome[1], only about 12,000 are characterized, with no data concerning protein localization of the remaining 60%--many of which have been reported amplified or overexpressed in breast cancer. Determining the localization of any one protein by traditional methods is an expensive and time-consuming process. However, a "feature" of membrane-bound and secreted proteins can be exploited to accurately determine their membrane-bound status on a large scale[2]. The mRNA transcripts of membrane-bound and secreted proteins are translated in polysomes bound to the endoplasmic reticulum (ER). Because their association with the ER makes them less dense, these membrane-bound polysomes (MBP) can be separated from their heavier cytosolic counterparts by sucrose gradient centrifugation[3]. Thus, a genomic approach utilizing this feature can be developed to distinguish which genes encode membrane-bound and secreted proteins. Combined with breast cancer expression and amplicon data[4-8], this should allow for the identification of potential novel drug targets and markers.

Objective/Hypothesis: We hypothesize that we can reliably separate membrane-bound polysomes from cytosolic polysomes, and evaluate each polysome fraction on microarrays to accurately predict the membrane-bound and secreted proteins in a breast cancer cell line. This data can then be compared with amplicon and expression data to identify novel potential therapeutic targets and markers.

Specific Aims: (1) Analyze the membrane-bound and cytosolic polysome fractions obtained from MCF7 cells on Affymetrix U95 GeneChips. (2) Determine the predictive ability of this data set against both known membrane-bound and cytoplasmic proteins, and generate an annotated database of proteins likely to be membrane-bound or secreted in MCF7 cells. (3) Identify genes encoding membrane-bound and secreted proteins that are known to be amplified, overexpressed, or differentially expressed in breast cancer.

Research Progress:

Task 1. **To analyze the membrane-bound and cytosolic fractions obtained from MCF7 cells on Affymetrix U95 GeneChips (Months 1-12):**

a. Extract membrane-bound and cytosolic RNA from the breast cancer cell line MCF7 and evaluate the quality of the separation by real-time PCR

$5x10^8$ MCF7 cells were grown in cell culture and gently lysed by Dounce homogenization in the presence of cycloheximide, a protein synthesis inhibitor, and Rnasin (Promega), a RNAse inhibitor. The lysate was separated by discontinuous sucrose density centrifugation in an ultracentrifuge at >25,000g for 5 hours. This would allow the less dense membrane associated polysomes to float to the top of the column, while the free cytosolic polysomes remained at the bottom. The column was then fractionated from a small hole in the bottom. The OD_{260} of each fraction was taken and used to form two pools, membrane-bound (MBP) and free cytosolic polysomes (FP). This fractionation has been repeated many times to ensure reproducibility and quality. A representative fractionation is shown in figure 1.

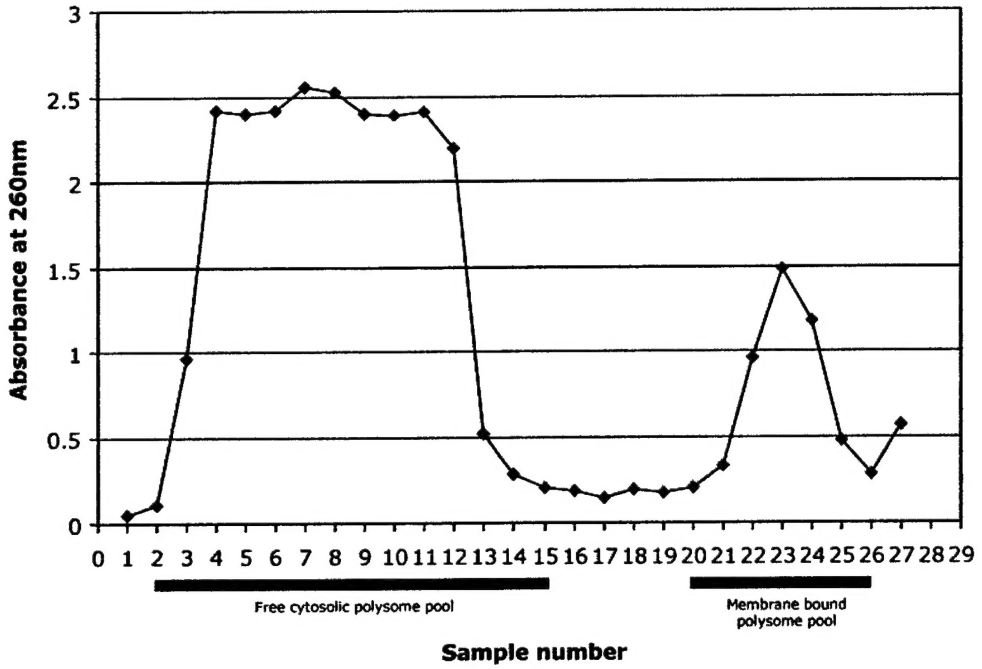

Figure 1. UV spectroscopy of fractions recovered from sucrose density gradient. Sample number corresponds to the order each sample was extracted from the bottom of the gradient. Samples 2-15 are pooled as the free polysomes, while samples 20-26 are pooled as the membrane-bound polysomes.

Although we have generated a large amount of high quality RNA from the cytosolic fraction after Trizol extraction, we have encountered some difficulty in obtaining enough high quality RNA from the membrane fraction for Affymetrix hybridization after RNeasy (QIAGEN) purification. We are currently in the process of troubleshooting the purification step, however we are confident that we will generate enough MBP RNA in our next fractionation to proceed. The quality of RNA was determined by OD_{260}/OD_{280} ratio and appearance on a 1% agarose gel. The RNA was quantified by spectrophotometry.

We do, however, have enough MBP RNA to evaluate test genes for enrichment in either fraction, by Realtime RT-PCR, as outlined in Task 1a of my statement of work. To generate cDNA, approximately 1μg of RNA from each pool was reverse-transcibed using Superscript II Reverse Transcriptase (RT) Kit in the presence of oligo $(dT)_{12\text{-}18}$ in a final 20μl reaction volume, per manufacturer's protocol, followed by Rnase H treatment.

We have used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a cytoplasmic control and junctional adhesion molecule (JAM) as the membrane control. These two genes were chosen because they were known to be abundantly expressed in MCF7 cells, according to SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE/), an online expression database. The data is summarized in table 1.

	JAM	GAPDH	JAM/GAPDH	GAPDH/JAM
Cytoplasmic Fraction	5.463333	652.5733	0.008	119.446
Membrane Fraction	2.113333	0.42	5.032	0.199

Table 1. Average Realtime RT-PCR results measuring differential gene expression in the membrane and cytoplasmic pools of RNA. See text for details.

These preliminary Realtime RT-PCR results indicate that these pools correspond to the biological differences we expect. As shown in table 1, JAM is significantly enriched in the membrane fraction, when compared to GAPDH, and GAPDH is significantly enriched in the cytoplasmic fraction, compared to JAM. Note that there is more JAM in the cytoplasmic fraction than the membrane fraction. This is does not necessarily mean that JAM is primarily found in the cytoplasm. Likewise, a 1 to 1 ratio does not necessarily mean that mRNA is found in equal amounts in each fraction. This is because mRNAs and polysomes may become dissociated from the ER during processing or cDNA synthesis may have proceeded at different rates in each pool. For the actual Affymetrix data, a test set of known genes that produce membrane-localized, secreted, and cytoplasmic proteins, must be used to determine what membrane/cytoplasmic ratios correspond to a probable membrane-bound or secreted protein. This is already outlined in Task 2, and is currently underway.

As soon as sufficient high quality MBP RNA is produced, complementary RNA (cRNA) made from each pool will be hybridized to Affymetrix U95 GeneChips, compared *in silico*, and a ratio of membrane/cytosol (m/c) will be determined for each gene. As stated above, known characterized proteins from published databases will be used to determine the accuracy of each m/c ratio in predicting membrane status. Genes predicted to be membrane-bound and secreted with a high confidence level will be compared to published and unpublished (from the mentor's lab) amplicon and expression data to identify potential drug targets and markers.

Key Accomplishments

- Reliably and reproducibly separated RNA from the MCF7 cell line by discontinuous sucrose density gradient ultracentrifugation into membrane bound and cytoplasmic pools.
- Evaluated the quality and quantity of the RNA in each pool by spectrophotometry and gel analysis
- Tested two genes, GAPDH and JAM, by Realtime RT-PCR and found that they were appropriately enriched in each pool.

Reportable Outcomes

- None, yet.

Conclusions

The work is progressing, with task 1a, the polysomal separation, nearly complete, but behind the schedule proposed in the statement of work for that task. Tasks 1b and 1c, which involve generating cRNA from the pools and hybridizing them to the Affymetrix Test array and Genechip will take much less time than originally budgeted, however, as the genomics facility on campus has become very experienced in hybridizing Affymetrix chips. The current turnaround time for hybridization to the Test array and Genechip is 3 weeks. This significant time savings should allow us to keep on schedule for the approved statement of work. The next steps after obtaining the hybridization data will be to validate the predictive power of this method on a set of test genes, and then identifying potential genes encoding membrane-bound or secreted proteins in the unknown set. These genes will then be compared to the genes found overexpressed or amplified in breast cancer in the literature and other sources.

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